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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Renner *et al.*	Art Unit: 1632
Appl. No.: 09/275,883	Examiner: R. Schnizer
Filed: March 25, 1999	Atty Docket: 1700.0020001/JAG/EEF
For: **Inducible Alphaviral Gene Expression System**	

Declaration Under 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

I, Marco Boorsma, do hereby declare and say:

1. I am a graduate of the University of Gronigen (The Netherlands), where I obtained the degree of Doktorandus (Drs.) in June 1998, and I was previously a research scientist at Cytos Biotechnology, the assignee of the above-captioned application.

2. The experiments described herein provide evidence that the pCYTts vector functions in a non-cytopathic, temperature sensitive manner in a variety of mammalian cell lines. Certain experiments described below employed the vector eCYTts, which is a derivative of pCYTts that contains a puromycin resistance marker under the control of a SV40 promoter. The vector eCYTts was non-cytopathic and temperature-sensitive in a variety of mammalian cell types. These assays were carried out as follows.

3. The cell lines BHK21 C13-2P, BHK21, CHO-K1, HEK 293, and HEK 293T were cotransfected using LIPOFECTAMINE 2000 (LF 2000) with (i) the vector eCYTts containing the coding sequence for secreted alkaline phosphatase (eCYTts-SEAP (Fig. 1)) and (ii) the marker construct pEGFP, encoding enhanced green fluorescent protein. At one day prior to transfection, cells were plated in their normal growth medium containing serum and without antibiotics; they were 90-95% confluent on the day of transfection. Suspension cells were prepared at a concentration of 1.5×10^6 cells per 6-wells (2 ml). If the normal growth medium was a medium other than DMEM, DMEM containing 10% fetal calf serum was substituted for the normal growth medium at two hours prior to transfection. For each well of cells to be transfected, 3.6 μg eCYTts-SEAP and 0.4 μg pEGFP were diluted into 250 μl OptiMEM I supplemented with glutamax.

4. For each well of cells, the appropriate amount of LF 2000 reagent was diluted into OptiMEM I supplemented with glutamax and incubated for 5 minutes at room temperature. For CHO-K1 cells, 12.5 μl of LF2000 were used; 10 μl of LF2000 were used for HEK 293 cells; and 15 μl of LF2000 cells were used for BHK21 cells. The diluted DNA was combined with the diluted LF2000 reagent by gentle mixing, and then incubated for 20 minutes at room temperature. The resulting solution then was added to each well of cells, covering the whole well, and the cells were incubated at 37°C. At 5 hours after transfection was initiated, the cell medium was replaced with 2 ml of normal growth medium, and the cells were further incubated at 37°C.

5. One day after transfection, the cells were split at the following ratios 1:4, 1:10, and 1:20, then incubated at 37°C. On the second day after transfection, the cells that were split

1:4 and 1:10 were shifted to 29°C in order to induce SEAP expression from the temperature-sensitive vector. The cells that were split 1:20 remained at 37°C for comparison.

6. At 5 days after transfection, the SEAP activity of the conditioned medium of each well was determined as follows. The conditioned media was heated to 65°C for 5 minutes. The media then was centrifuged at 14,000 rpm for 30 seconds, and 50 µl of each supernatant was transferred into a 96-well plate. A 50 µl aliquot of FAST™ p-Nitrophenylphosphate was added, and absorbance was measured at 405 nm every 30 seconds for 20 minutes at 37°C using a Benchmark microplate reader. After plotting the data, the slope of the linear part of the curve was determined, giving the SEAP activity in 50 µl of conditioned medium. The SEAP activity of the entire well was determined by multiplying the activity in 50 µl by 40.

7. The cells then were harvested and split into two aliquots. In one aliquot, viable cells were counted using a CEDEX cell counter. The second aliquot was used for determining the percentage of transfected cells using a FACS Calibur. The SEAP activity per transfected and per viable cell was calculated.

8. The obtained data indicate that the vector eCYTts-SEAP, a derivative of pCYTts, is non-cytopathic and induces temperature-sensitive gene expression. Table 1, below, shows that SEAP activity was detected in the supernatants of cell cultures that had been induced at 29°C to express SEAP. SEAP expression was measured in BHK21, BHK21-C13-2P, HEK 293, HEK293T, and CHO-K1 cells at 7 days post-transfection. While SEAP activity was detected in supernatants of cells that were temperature-shifted to 29°C, SEAP activity was not detected

supernatants of the same cotransfected cells that were maintained at 37°C, indicating that the vector functioned in a non-cytopathic, temperature-sensitive manner.

Table 1: Cells transiently cotransfected with eCytTS SEAP and pEGFP

Cell Line	SEAP Activity of Supernatant After 64 hrs at 29°C [mOD/(min*1000,000 cells)]	Viable Cells (100,000 cells)	Viability (%)
BHK21 C13-2P	0.49	5.54	88.5
BHK21	0.28	39.64	94.9
HEK293t	0.16	25.37	95.4
HEK 293	0.20	8.18	92.2
CHO-K1	0.13	4.62	94.7

9. In similar experiments, the vectors eCYTts and pCYTts were used to obtain inducible expression of GFP in various mammalian cells. The vector eCYTts-GFP was used to express GFP in BF cells in a non-cytopathic, temperature-sensitive manner. As shown in Fig. 2, transfected cells that were induced at 29°C for 48 hours expressed GFP, as detected by fluorescence microscopy. Likewise, the vector eCYTts-AID56-GFP was used to express an AID56-GFP fusion protein in C2C12 myoblasts in a non-cytopathic, temperature-sensitive manner. AID56-GFP is a fusion protein of the amyloid-β precursor protein intracellular protein and green fluorescent protein. As shown in Fig. 3, expression from the eCYTts vector was induced at 29°C for 48 hours, and expression of the AID-GFP fusion protein was detected by fluorescence microscopy. As shown in Fig. 4, GFP expression was also induced and detected in CHO-K1 cells that were stably transfected with CYTts-GFP and induced at 29°C for 10 days.

Additionally, inducible GFP expression was detected in COS-7 cells that had been stably transfected with pCYTts-GFP (data not shown).

10. Further evidence that the vector pCYTts is non-cytopathic is provided by the enclosed Fig. 5. In this assay, HEK 293 cells were transfected with the vector pMut1-GFP-IRESpuro (Fig. 5A). This vector is similar to pCYTts, but lacks the ts6 mutation that renders the polymerase activity temperature sensitive. Cells transfected with pMut1-GFP-IRESpuro were grown under puromycin selection for 2 weeks (Fig. 5B), and essentially all of the cells in the clone produced GFP, as detected by fluorescence microscopy (Fig. 5C). Thus, this experiment shows that the pCYTts vector is non-cytopathic, contrary to the findings of Agapov *et al., Proc. Natl. Acad. Sci., USA* 95:12989-12994 (1998).

11. Still further evidence that the pCYTts vector can be used in a non-cytopathic, temperature-sensitive manner is provided by the enclosed publication by Boorsma *et al.*, *Nature Biotechnology* 18:429-432 (2000). The pCYTts vector was used to obtain inducible expression of genes encoding SEAP, GFP, β-interferon, and erythropoietin in CHO-K1 cells, COS-7 cells, C2C12 myoblasts, and BHK cells as well as to obtain inducible expression of genes encoding RIP death domain in BHK cells.

12. In sum, the data described herein support the assertion that the vector pCYTts can be used to obtain inducible expression of a gene of interest in a non-cytopathic, temperature-sensitive manner.

13. THAT, I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this document or any patent associated herewith.

Date 27/07/01

Dr. Marco Boorsma